

The use of the scanning electron microscope (SEM) to reconstruct the ultrastructure of sporoderm

Natalia Zavialova* and Eugeny Karasev

A.A. Borissiak Paleontological Institute, Russian Academy of Sciences, Profsoyuznaya 123, Moscow 117647, Russia

Scanning electron microscope (SEM) data can help in the interpretation of transmission electron microscope (TEM) ultrathin sections and reconstruction of the three-dimensional inner structure of large palynological objects like megaspores. For a SEM study of the inner structure of fossil megaspores, we tried three variants of embedding media: a water solution of glycerine and gum arabic, a water solution of sucrose and polyvinylpyrrolidone, and a mixture of epoxy resins. Semithin sections of fossil megaspores were made, the embedding medium was removed from the sections and they were observed under SEM. Epoxy mixture as an embedding medium and Maxwell's solution as a solvent turned out to be the most appropriate for our purposes. The most suitable way of processing is to embed the object, cut it by turns in semithin and ultrathin sections, and study them with SEM and TEM correspondingly. A combination of SEM and TEM data results in a more profound reconstruction of the inner structure of sporoderms. We used as test objects dispersed megaspores of a supposed lycopsid affinity identified as *Maexisporites rugulaeferus* Karasev et Turnau 2015 and *Otynisporites tuberculatus* Fuglewicz 1977. The materials studied are from the Lower Triassic and Upper Permian of the Russian Platform.

Keywords: SEM; semithin sections; sporoderm ultrastructure; 3-D reconstructions; fossil megaspores; TEM

1. Introduction

Transmission electron microscopy (TEM) has remained the most powerful and irreplaceable tool in studies of sporoderm ultrastructure. However, there are palynological objects that allow the scientist to reconstruct their three-dimensional (3-D) ultrastructure from two-dimensional (2-D) TEM ultramicrographs with greater difficulty than others. In particular, megaspores are too large in terms of TEM: one needs to take up to several dozens of individual photos and sew them into a composite image before the observation of a single ultrathin section becomes possible. One cannot cut the entire megaspore, but only a portion of it. Therefore, there is a danger that areas with significant peculiarities will remain unstudied. The ultrastructure of the megaspore sporoderm can differ within a single specimen, dependent on the distance from the surface to the megaspore lumen and/or from the poles to the periphery of the spore. The sporoderm can be composed of different structural elements in its different regions, and even similar structural elements can vary in dimensions and orientation and denser or looser mutual arrangement. Some structures occupy very limited areas and can be overlooked.

Our experience has shown that observation under scanning electron microscope (SEM) of mechanically ruptured megaspores can be very helpful for understanding the inner structure, complementing and

clarifying TEM data. For example, Zavialova & Turnau (2012) studied Devonian megaspores of *Grandispora ciliata* Fuglewicz et Prejbisz 1981. Ultrathin sections at the junction between the inner body and the outer envelope were difficult to interpret. In this area, some individual structural elements appeared as laminae, whereas others were circles implying that they are either granules or cylinders (Plate 1, figures 1, 2; Figure 1C). We did not understand how the same structural elements can be both flat and cylindrical (or granulate). The problem was solved with help of a single SEM image showing a mechanically ruptured sporoderm of the proximal pole (Plate 1, figures 3, 4). The elements, which mysteriously varied in outline, were finally understood. They were small flat tabular elements with appendages (Plate 1, figure 5; Figure 1C). In TEM sections, the body of such elements appeared as short laminae, and the appendages either as granules (if cut transversely) or as short laminae (if cut longitudinally).

To study the inner structure of megaspores with SEM, we followed the advice given by Prof. Lugardon (pers. comm.): to cut slightly wetted megaspores into halves with a razor blade and observe the obtained sections sideways on a tilted SEM stub (e.g. Zavialova & Turnau 2012, plate II, figures 11, 13). However, a SEM study of several semithin sections or series of such sections seemed more promising. Sections made with the help of an ultramicrotome could be orientated less

*Corresponding author. Email: zavial@mail.ru

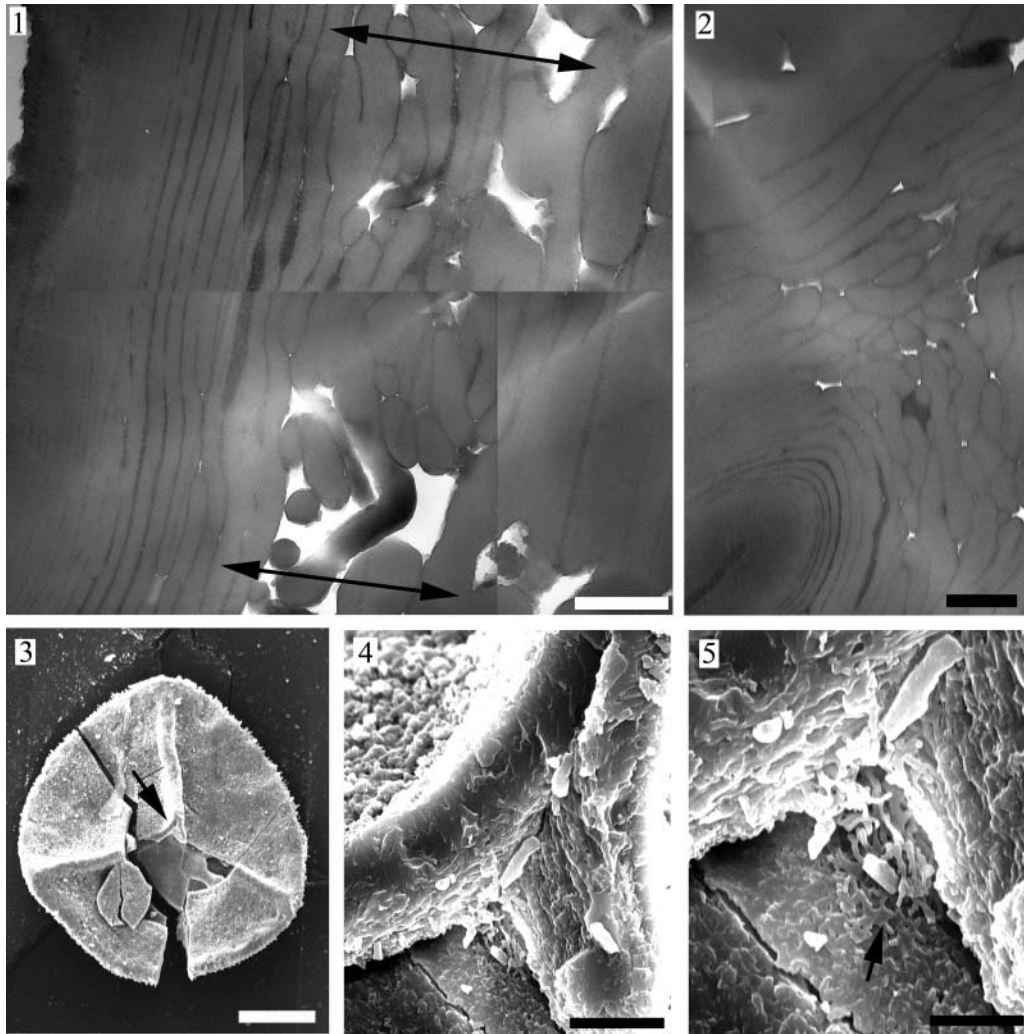


Plate 1. Inner structure of megaspores of *Grandispora ciliata* Fuglewicz et Prejbisz 1981 in scanning electron microscope (SEM) and transmission electron microscope (TEM) images. The illustrations are reproduced from Zavialova & Turnau (2012). 1. The inner body and innermost region of the outer envelope of the proximal wall. The lumen is visible in the upper left. Note various outlines of the sectioned units. The area of junction between the inner body and the outer envelope is marked with arrows. 2. Inner equatorial region. Note variable outlines of the sectioned structural elements at junction of the inner body and outer envelope. 3. Proximal view of a broken megaspore; arrow indicates the position of the detail shown in figures 4, 5. 4. Detail of image in figure 3, showing fractured labrum of the trilete mark. 5. Enlargement of figure 4. Tabulate units with appendages (arrow) are present on the exposed surface of the inner body (compare with Figure 1C). Scale bars: 1 = 0.5 μm ; 2 = 0.67 μm ; 3 = 100 μm ; 4, 5 = 10 μm .

accidentally than manual sections. In addition, the stub cannot be tilted perpendicularly; therefore, it is impossible to accomplish correct measurements of the thicknesses of the sporoderm and its sublayers in manually sectioned spores.

We searched for a method to make such sections. An embedding medium was needed, which could be easily removed from sections in order to observe only sporoderm under SEM. Although one can observe embedded biological objects directly on a block surface, problems appear of low contrast, charging and lower resolution. These problems are solvable and even a series of sections can be obtained, e.g. by means of serial block-face SEM (Denk & Horstmann 2004), but it seemed to us that a study of un-embedded

semithin sections observed with a conventional SEM is a shorter and easier way to achieve our aims, since it does not need either complicated handling or even modifications of the microscope.

First, we tried a water solution of gum arabic and glycerine. Earlier, it was successfully used to embed modern pollen, cut them with a microtome, and study the sections under light microscope (LM) (Leins 1968) or SEM (Hideaux & Marceau 1972; Audran & Masure 1977). Another variant we attempted was proposed by Chentsov et al. (1973), who studied mitotic chromosomes of tulips under TEM in sections 1–3 μm thick. A drop of a water mixture of sucrose and polyvinylpyrrolidone (PVP) was placed on an epoxy block. The material under study was sunken into this drop, hardened

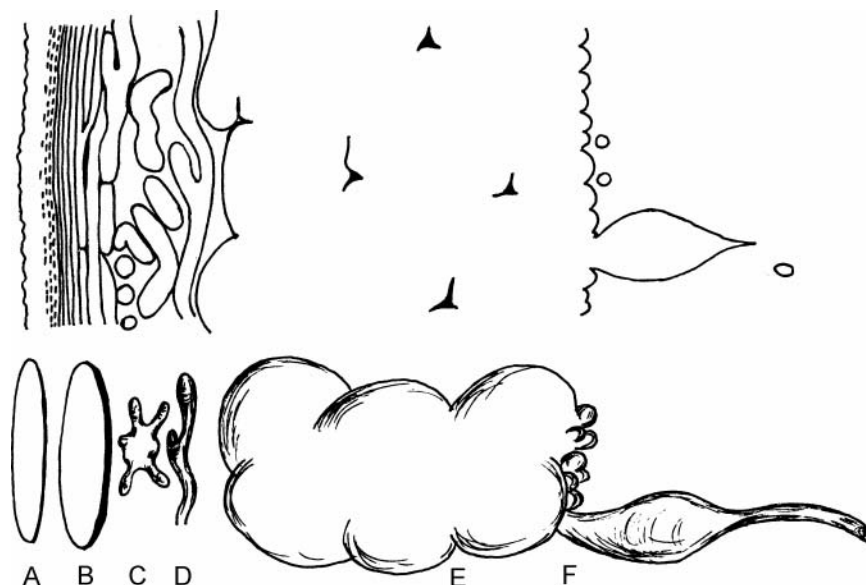


Figure 1. Diagrammatic representation of the wall of *Grandispora ciliata* Fuglewicz et Prejbisz 1981 (top), and its constituent parts (bottom): A. thin laminate unit; B. thick laminate unit; C. tabulate unit with appendages; D. cylindrical forking unit; E. fused globular unit; F. granular unit. The figure is reproduced from Zavialova & Turnau (2012), available at <http://www.sciencedirect.com/science/article/pii/S0034666708001073>

overnight at room temperature and cut into semithin sections with an ultramicrotome equipped with a dry glass knife. The sections were taken from the knife edge with an eyelash and placed in a drop of water, where the medium was washed out. The sections were transferred onto grids and observed under TEM. In the absence of the medium, a better contrast was achieved without chemical staining. The last variant that we tried was embedding objects into an epoxy mixture as for a TEM study, preparation of semithin sections and removal of the epoxy resin from the sections, after Maxwell (1978).

Removal of embedding medium from sections has been earlier accomplished for various purposes, for example, in cytology (Capco et al. 1984), immunohistochemistry (Vidal et al. 1995) and morphological studies of insects (Gorb 2000). We attempted it to study the sporoderm ultrastructure of fossil megaspores.

2. Materials

We took as test objects dispersed megaspores of a supposed lycopsid affinity from the Lower Triassic and terminal Upper Permian deposits of the Russian Platform: *Maexisporites rugulaeferus* Karasev et Turnau, 2015 and *Otynisporites tuberculatus* Fuglewicz 1977 (Karasev & Turnau 2015). Our electron-microscopical study of the inner structure of Upper Permian/Lower Triassic megaspores is still in progress and will be published later in full detail; in this paper, we evaluate the benefits and shortcomings of the SEM method of study of semithin sections of fossil megaspores.

Specimen PIN #4820/804 of *O. tuberculatus* was collected from terminal Late Permian deposits of the Nedubrovo locality. The Nedubrovo locality is exposed in a series of large outcrops on the left bank of the Kichmenga River of the Vologda region. The stratigraphic position of the locality is regarded as the upper part of the Changhsingian (Lozovsky et al. 2014). Specimens identified as *M. rugulaeferus* Karasev et Turnau, 2015 (PIN #5529/127 and 5529/105) and *O. tuberculatus* (PIN #5529/120) were collected from the Sholga locality. The Sholga locality is a natural exposure situated on the left bank of the Yug River, 200 m upstream from the ferry pier in Sholga village (Kirov Region, Podosinovskii District). The stratigraphic position of these deposits is regarded as a lower part of the Induan (Yaroshenko & Lozovsky 2004).

Collections PIN #5529 (Sholga locality) and PIN #4820 (Nedubrovo locality) are kept at the A.A. Boris- siak Paleontological Institute, Russian Academy of Sciences, Moscow.

3. Methods

The megaspores were isolated from the encompassing sediments by disintegration in water followed by treatment with hydrochloric acid (HCl), again water and finally by hydrofluoric acid (HF) to remove the silica component. The megaspores were picked from a Petri dish with a needle and mounted on SEM stubs to study their general morphology. This was accomplished under

a TESCAN VEGA-II XMU SEM (accelerating voltage 30 kV) at the A.A. Borissiak Paleontological Institute.

The megaspores were removed from SEM stubs. *Otyinosporites tuberculatus* (specimen PIN # 5529/120) was studied with TEM directly after SEM and finally again in SEM in resinless sections; *O. tuberculatus* (specimen PIN #4820/804) was further studied in SEM only in sections prepared with PVP-sucrose medium; and *Maexisporites rugulaeferus* Karasev et Turnau, 2015 (specimen PIN #5529/127) was studied in TEM and in resinless sections in SEM. *M. rugulaeferus* (specimen PIN # 5529/105) was used to illustrate the general morphology.

For TEM, the megaspores were embedded in a mixture of epoxy resins [Epon-812, dodecenyl succinic anhydride (DDSA), methyl nadic anhydride (MNA), and an accelerator as 17:15:8:1 volume ratios] for 48 h at 60 °C. Sectioning was accomplished with a Leica EM UC6 ultramicrotome at the A.A. Borissiak Paleontological Institute. Sections 70 nm thick were observed unstained under a Jeol 100B TEM (accelerating voltage 80 kV) at the Electron Microscope Laboratory of the Lomonosov Moscow State University.

For embedding, we first tried a mixture of 12 mL distilled water, 4.4 mL glycerine and 11 g gum arabic (Hideaux & Marceau 1972). However, we failed to make a suitable solution: air bubbles inevitably entered the medium during mixing, and neither time nor changed proportions, nor heating, nor degassing helped us to obtain a medium without bubbles which could have been suitable for embedding.

Second, we tried the medium proposed by Chentsov et al. (1973). We dissolved 1.15 g PVP and 1 g sucrose in 6.2 mL distilled water. A drop of this medium was placed on an epoxy block; a megaspore was sunk into the drop and hardened overnight at room temperature and a pyramid was made with a razor blade (Figure 2, 1c–1f). We obtained PVP-sucrose blocks of low quality. At first, hardened drops were too soft and weakly attached to epoxy blocks. Soft sections were difficult to detach from the block after sectioning; after several sections, the knife tore out the entire PVP-sucrose block from the epoxy block. One of the authors of the method, Prof. Polyakov, advised us to use plexiglass for blocks on which PVP-sucrose drops will be placed and to treat their surface with dichloroethane for better adhesion of PVP-sucrose drops (Figure 2, 1a, 1b). In this way we obtained blocks which we managed to cut with a dry diamond knife (at a Leica EM UC6 ultramicrotome) at an increased speed of sectioning (1.00 mm/s) into semithin sections 1 µm thick, which we later observed with SEM (Figure 2, 1g). However, at each embedding, blocks differed in hardness and elasticity. Often, we obtained blocks that were too brittle. Moreover, blocks

were quite heterogeneous in hardness, dependent on the distance from the surface. We varied temperature (during hardening and cutting), time of hardening and relative amounts of the components, but did not improve the results significantly. Most often, blocks were hard at the surface and soft near the object, but cuttable. After cutting, sections were removed from the edge of the knife with an eyelash attached to a toothpick and placed in a drop of distilled water in a cavity slide (Figure 2, 1g, 1h). The medium was dissolved in water; the sections were cleaned in several water drops in cavity slides (Figure 2, 1i). We prefer cavity slides manufactured for dentistry to those designed for sero-diagnostic tests, since they are of a greater diameter and depth, and there is enough space to add liquids. They are better than watch glasses, because they have a flat inner surface and are mountable on the stage of a transmitted light microscope and one can check the objects under low magnification of the microscope.

We used a small cover glass to mount sections on a SEM stub. We attached it temporarily to a glass slide with pieces of plasticine (Figure 2, 3a). A very small drop of water was placed on the cover glass with a perfect loop, which is a tool designed for picking up ultrathin sections from water and transferring them onto grids (<http://www.diatome.ch/en/products/perfectloop.asp>; Figure 2, 2c). The sections were transferred into such drops with an eyelash (Figure 2, 3b, 3c); the water evaporated (Figure 2, 3d). We attached to a large SEM stub a piece of double-sided sticky tape, which was slightly larger than the cover glass. The cover glass with dried sections was detached from the glass slide and attached to the stub. For easy screening, we drew circles with a marker around sections (Figure 2, 3e, 3f). A survey photo of the stub was made under a dissecting microscope before sputtering, since the sections are very flat and difficult to find under low magnifications of SEM. The stub was coated with gold for 3 min. The sections were observed under a TESCAN VEGA-II XMU SEM (accelerating voltage 30 kV) at the A.A. Borissiak Paleontological Institute (Plate 4, figures 3, 5, 7).

The last variation that we tried (Figure 2, 2a–2f) was removal of epoxy from sections after Maxwell (1978). A series of sections (1 µm thick) was made into water with the help of a Leica EM UC6 ultramicrotome, equipped with a diamond knife (Figure 2, 2b). The sections were transferred with the help of a perfect loop into a cavity slide (Figure 2, 2c, 2d). The success of this transfer was checked under a transmitted light microscope. After all series of sections that were planned to make were placed in cavity slides and protected against air dust with cover glasses temporarily attached with pieces of plasticine, we dissolved two pellets of potassium hydroxide (KOH) in 2 mL of absolute methyl alcohol and 0.5 mL of

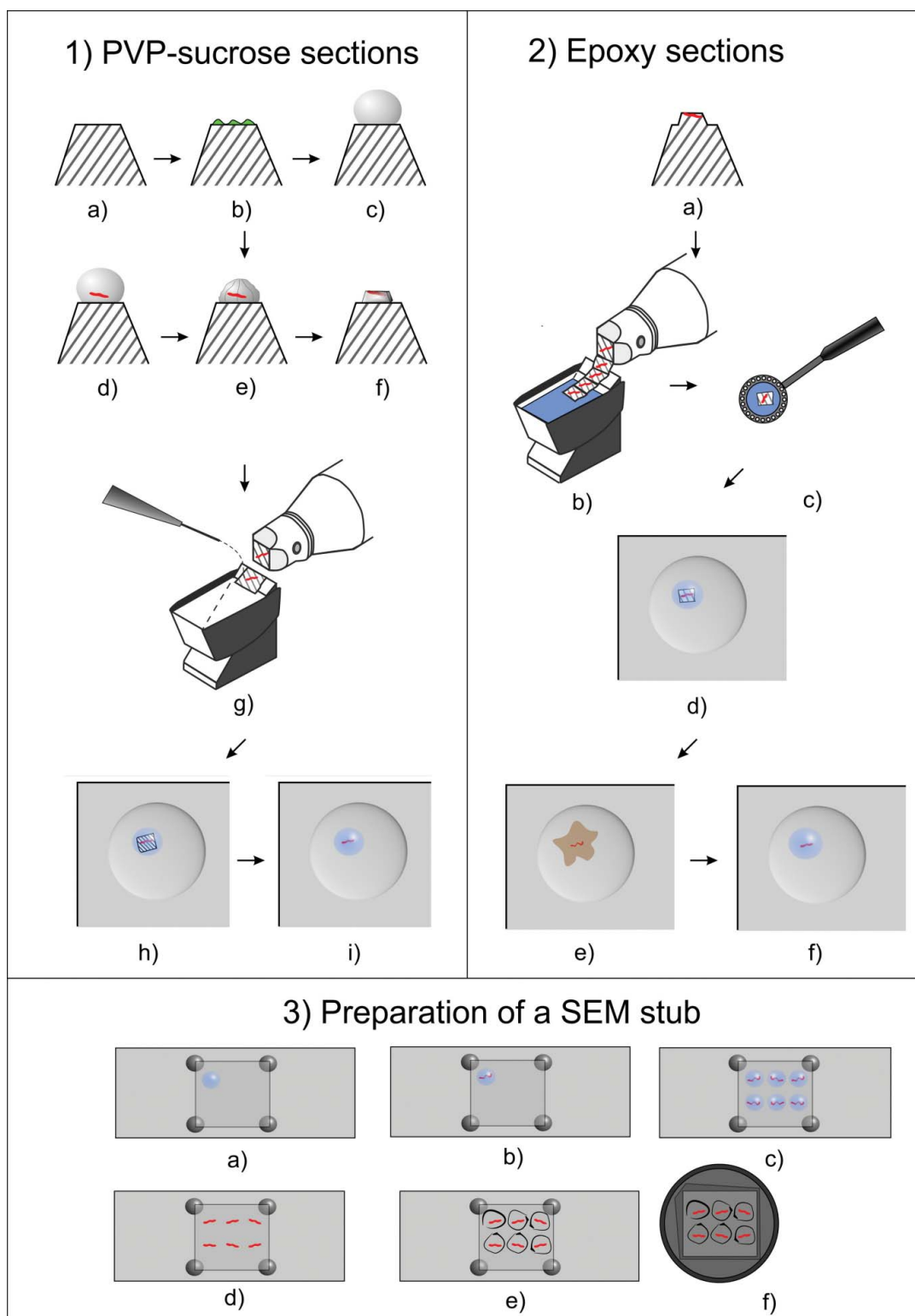


Figure 2. Main stages of preparation for a scanning electron microscope (SEM) study of semithin sections. 1a–1f. Preparation of sections using polyvinylpyrrolidone (PVP)-sucrose medium. a. A plexiglass block is prepared. b. The surface of the block is treated with dichloroethane. c. A PVP-sucrose drop is placed on the block. d. A megaspore is sunken into the drop. e. The drop hardens overnight. f. A pyramid is made. g. Sections are made with a dry knife and removed from its edge with an eyelash. h. The sections are placed in a drop of water. i. The medium is dissolved, and the sections are cleaned in several water drops. 2a–2f. Preparation of sections using epoxy resin. a. A megaspore is embedded in epoxy mixture; a pyramid is made. b. Sections are made into water. c. Sections are transferred with a perfect loop. d. Sections are placed on a cavity glass. e. The resin is dissolved. f. The sections are washed in several drops of water. 3a–3f. Preparation of a SEM stub. a. A cover glass is attached to a glass slide, and a small drop of water is placed on the cover glass. b, c. Sections are transferred into drops. d. Water evaporates. e. Circles are drawn around sections. f. The cover glass with sections is attached to a SEM stub.

propylene oxide. The mixture was stirred on a magnetic stirrer for about 15 min.

We removed the cover glass from a cavity slide and placed several drops of the mixture, using a glass rod, over the dried sections (Figure 2, 2e). The dissolution of resin is a visible process, and we controlled it under a dissecting microscope. We cautiously disturbed with an eyelash those sections that were too tightly attached to the glass surface and did not detach by themselves. Since the solution evaporates rapidly, in some cases, we added several more drops once or twice, but usually the resin disappeared in a few minutes without the addition of more solution. Epoxy sections are flat. When the epoxy medium is removed from the sections, they often tend to curl (some of them we managed to smooth out later with eyelashes; others were discarded). After the resin was removed, sections were transferred with an eyelash into a drop of distilled water in a cavity slide (Figure 2, 2f). They were washed in several drops of distilled water in cavity slides. We transferred them from drop to drop with an eyelash. However, if sections are very long, too many of them get mechanically damaged, and for such sections we cautiously removed water with a piece of filter paper, added a new drop of water, and repeated the procedure many times. We checked that the sections were clean under a transmitted light microscope. We prepared the SEM stub in the same way as we did for PVP-sucrose sections (Figure 2, 3a–f). The sections were observed under the same SEM (Plate 2, figure 2; Plate 4, figures 2, 4, 6, 8).

4. Results

Maexisporites rugulaeferus (Plate 2, figures 1–6; Figure 3). TEM shows the outer exospore of the sporoderm, which is composed of units that are variable in outline in sections (Plate 2, figures 1, 3). Judging from TEM images, they can be branches, or laminae, or granulae. SEM images of semithin sections unequivocally show that the outer exospore is composed of branching elements (Plate 2, figure 2). What SEM fails to reveal is the ultrastructure of the inner exospore. Only a homogeneous thick layer is visible, but no lamellation is discernible in semithin sections under SEM (Plate 2, figure 2, bottom of the figure), whereas ultrathin sections under TEM demonstrate distinct lamellation in this region (Plate 2, figure 4).

Otynisporites tuberculatus (Plate 3, figures 1–4, Plate 4, figures 1–8; Figure 4). With TEM, we discovered peculiar structures in the inner exospore, which we finally interpreted as inner papillae in terms of Grauvogel-Stamm & Lugardon (2004). One to several short thickenings of this layer were observed in some sections (Plate 3, figure 1), whereas consequent sections

of the series contained only one thickening or no thickening. We were not completely sure that these structures were not artifacts. During fossilisation, sporoderms flatten to a different degree within the same megaspores, and areas that are equal in thickness in a living megaspore can differ in thickness in a fossil megaspore. In addition, the sections are not strictly perpendicular. Therefore, some areas of an irregularly flattened megaspore, which were obliquely cut, can appear in sections thicker or thinner than they really are. However, the structures under consideration were detected in many sections; therefore, though the probability that they were artifacts existed it was low. One more question remained: what is the shape of these structures?

SEM answered both questions. It confirmed that these structures did exist (regardless of the direction of the section) and showed that most probably they can be described as inner papillae. Elements that are scattered over the inner surface of the basal lamina and appear in ultrathin sections as granulae are clearly understood in semithin sections under SEM as continuous elements forming a mesh (compare Plate 3, figures 2, 4 and Plate 4, figure 6). On the other hand, the comparison between SEM and TEM images of these inner papillae shows not only benefits but also shortcomings of SEM. The papillae seem homogeneous in SEM images, but TEM images at a high magnification demonstrate their lamellate nature (Plate 3, figure 4). However, being aware of the lamellate nature of papillae based on TEM data, one can remark some indices of it in semithin sections as well (Plate 4, figure 8).

5. Discussion

Attempts have been previously made to study the inner structure of modern pollen and spores by means of SEM (e.g. Audran & Masure 1977; Morbelli & Rowley 1999; Morbelli et al. 2003a, 2003b). Thus, Audran & Masure (1977) published microtome semithin sections of pollen of modern cycads studied under SEM. The characteristically cycadalean ectexine of regular elongated alveolae is clearly visible in sections of pollen of some taxa (e.g. *Dioon edule*; Audran & Masure 1977, plate 14), but nearly undetectable in others (e.g. *Zamia fisheri*; Audran & Masure 1977, plate 13, figure 7), though it was unequivocally shown by TEM (Audran & Masure 1977, plate 9). In their studies of *Selaginella*, Morbelli & Rowley (1999) and Morbelli et al. (2003a, 2003b) observed under SEM megaspores which were cut in half with a razor blade. The inner structure was clearly visible in 3-D; obtained data were analysed in combination with LM and TEM data.

The inner structure of fossil palynological objects has also been observed with help of SEM (e.g.

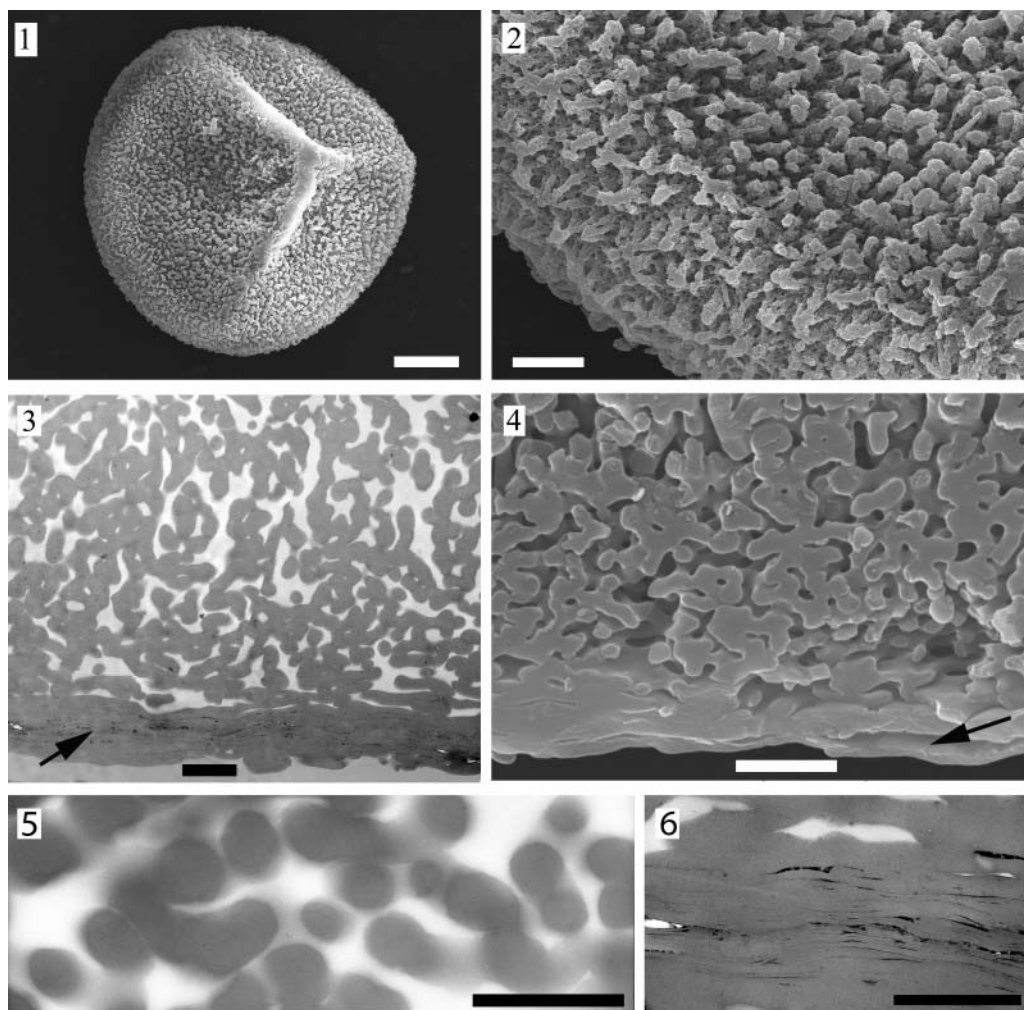


Plate 2. Inner structure of megaspores of *Maexisporites rugulaeferus* Karasev et Turnau, 2015 in SEM and TEM sections. 1. Proximal view of a megaspore, specimen PIN # 5529/105. 2. Enlargement of the surface of the megaspore shown in figure 1. 3. Area of an ultrathin section showing the inner portion of the outer exospore of the proximal side and fused inner exospore of the proximal and distal sporoderms. Constructing elements of the outer exospore vary in outline and directions. The gap at the bottom of the figure is a distal cavum. Arrow indicates the position of an obliterated lumen. TEM. Specimen PIN # 5529/127. 4. Area of a resinless section that corresponds to the section shown in figure 3. Arrow indicates the position of an obliterated lumen. SEM. Specimen PIN # 5529/127. 5. Predominantly circular outlines of sectioned units constructing the outer exospore. TEM. Specimen PIN # 5529/127. 6. Lamellate inner exospore of the proximal and distal sporoderms, pressed to each other. TEM. Specimen PIN # 5529/127. Scale bars: 1 = 50 μm ; 2 = 10 μm ; 3–5 = 1 μm ; 6 = 2 μm .

Meyer-Berthaud 1986; Meyer-Berthaud & Galtier 1986; Snigirevsky et al. 2007; Villanueva-Amadoz et al. 2012; Zavialova & Turnau 2012). Meyer-Berthaud (1986) and Meyer-Berthaud & Galtier (1986) illustrated mechanical ruptures of prepollen of Carboniferous seed ferns. However, constructing units are too densely situated to show the 3-D organisation correctly, as was shown by a comparison with TEM ultrathin sections of related taxa (Orlova et al. 2009). Snigirevsky et al. (2009, plate XVI, figures 9, 10) illustrated ruptured Devonian microspores, providing information on the inner structure of the sporoderm, though the authors interpreted it erroneously. Villanueva-Amadoz et al. (2012) used a dual-

beam focused ion beam scanning electron microscope (FIB-SEM) to visualise the 3-D inner structure of fossil isoetalean microspores and dispersed angiosperm pollen grains, and got promising results (e.g. Villanueva-Amadoz et al. 2012, figure 2D, E).

Semithin sections are more suitable for revealing the inner structure of palynological objects than mechanical ruptures and razor blade sections, because we can orientate semithin sections and obtain numerous sections and sections of certain desired regions. Unlike ruptures and manual sections, semithin sections are situated horizontally on stubs: we can accomplish correct measurements of the sporoderm and its

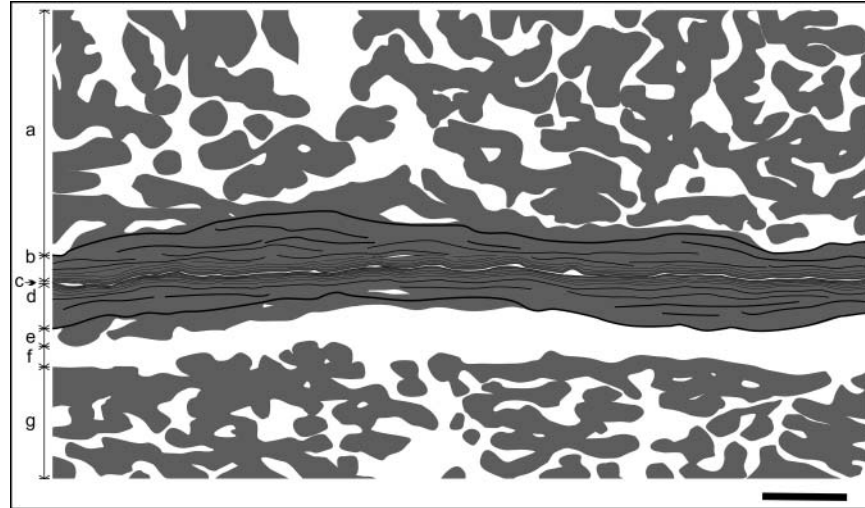


Figure 3. Diagrammatic representation of the inner portion of the sporoderm of megaspores of *Maexisporites rugulaeferus* Karasev et Turnau, 2015. Compare with Plate 2, figures 3, 4, 6. The proximal face is to the top of the figure. From the top to the bottom one can see: a. branching elements of the inner portion of the outer exospore of the proximal side; b. lamellae of the inner exospore fused to the outer exospore and becoming thinner towards the lumen; c. an obliterated lumen of the megaspore; d. lamellae of the inner exospore of the distal side e. several elements of the outer exospore fused to the inner exospore; f. a distal cavum within the outer exospore; and g. branching elements of the outer exospore. Scale bar 1 μm .

sublayers; in addition, the entire surface of the section is evenly illuminated and we can take images of good quality. For example, we were forced to over-illuminate a ruptured megaspore to discern structural units

situated in deep sublayers of the sporoderm (Plate 1, figures 4, 5), but structural elements of both outer and inner sublayers of the sporoderm are clearly visible in one and the same evenly illuminated semithin section

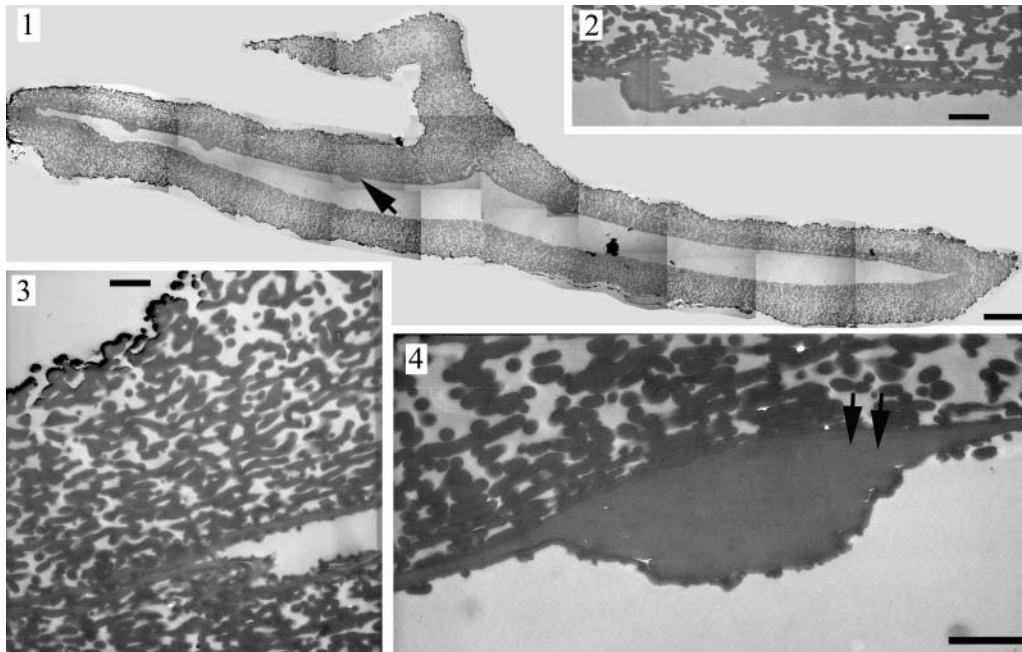


Plate 3. Inner structure of megaspores of *Otynisporites tuberculatus* Fuglewicz 1977, TEM. Specimen PIN # 5529/120. 1. Composite image of a section in the area of the proximal scar. Proximal face is to the top of the figure. Several inner papillae are visible proximally (arrow points to one of them). 2, 4. Inner papillae. Lumen is to the bottom of the figures. Fused lamellae are weakly discernible within the papilla (arrows). A continuous basal lamina covers the inner surface of the papilla; units that are circular in sections are scattered over the inner surface of the basal lamina. 3. Section in the lateral region of the sporoderm. Structural elements vary in outline and directions. A black outer contour is a remnant of SEM coating. Scale bars: 1 = 10 μm ; 2–4 = 1 μm .

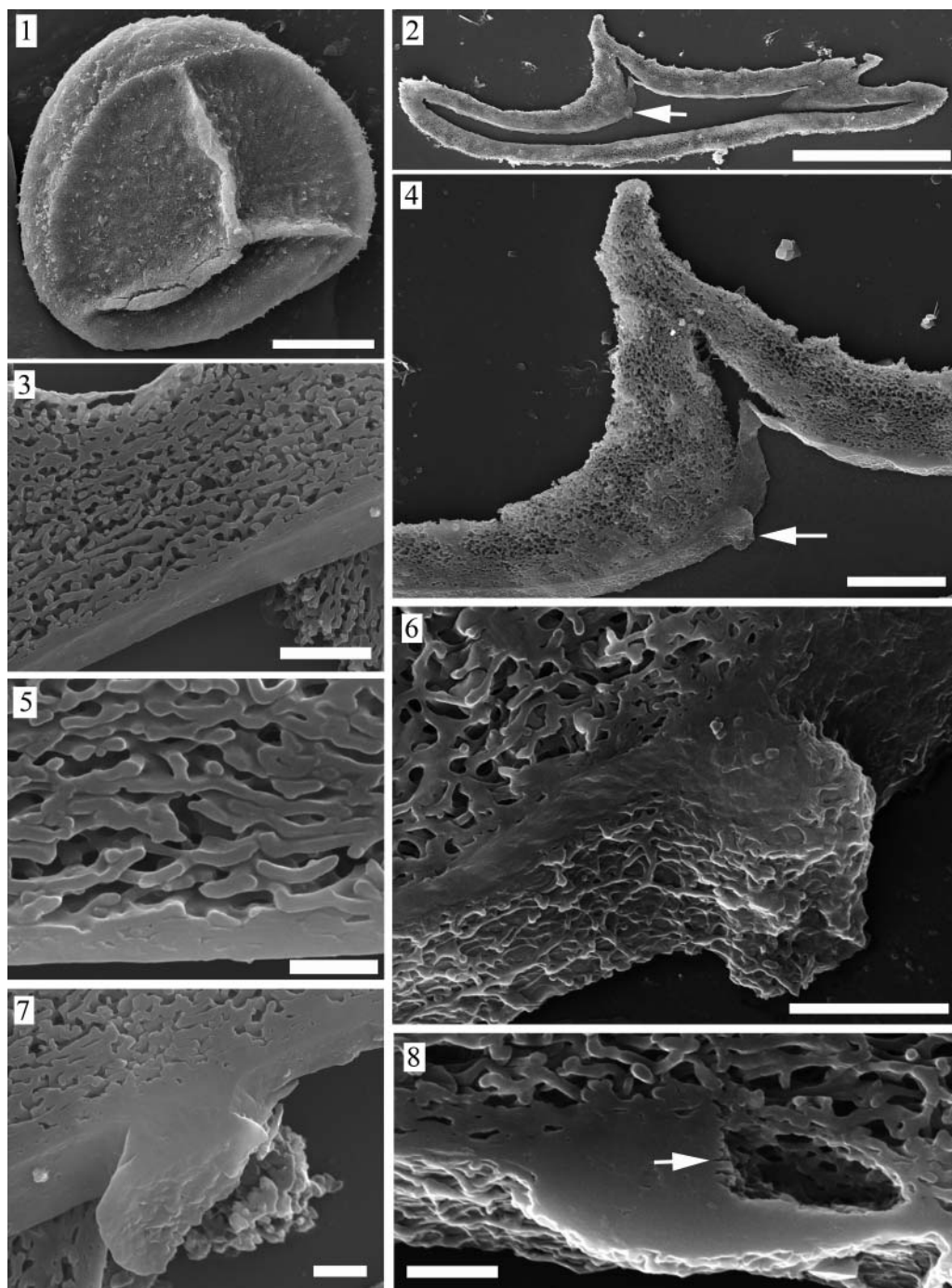


Plate 4. Inner structure of megaspores of *Otyinsporites tuberculatus* Fuglewicz 1977, SEM. 1. Proximal view of a megaspore, specimen PIN #5529/120. 3, 5, 7. Sections prepared with polyvinylpyrrolidone (PVP)-sucrose, specimen PIN # 4820/804. 2, 4, 6, 8. Sections prepared with epoxy resin, specimen PIN #5529/120. 2. A semithin section of a megaspore; two rays of the proximal scar are cut. Arrow indicates the position of the enlargement shown in figure 4. 3, 5. Areas of a semithin section of the sporoderm. The lumen is to the bottom of the figures. 4. Enlargement of figure 2, proximal area; arrow points to an inner papilla. 6. Inner papilla. Enlargement of figure 4. Compare with Plate 3, figure 4: SEM shows that circular elements scattered over the inner surface of the basal laminae represent a continuous mesh. 7. Inner papilla. 8. Inner papilla. Compare with Plate 3, figure 2. There is a slight indication (arrow) that the papilla is formed by fused lamellae. Scale bars: 1, 2 = 100 μm ; 3, 6 = 5 μm ; 4 = 20 μm ; 5, 7, 8 = 2 μm .

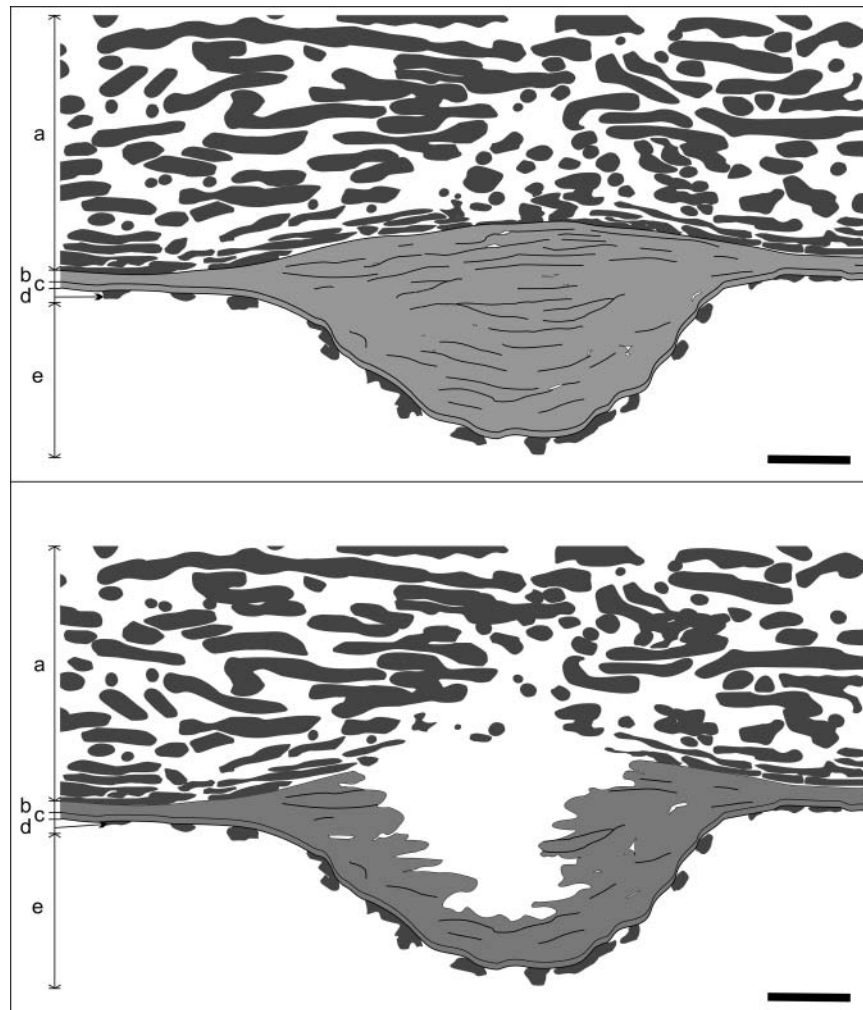


Figure 4. Diagrammatic representation of the sporoderm ultrastructure of megaspores of *Otynisporites tuberculatus* Fuglewicz 1977. A portion of the proximal sporoderm is shown. The upper part of the figure is a peripheral area of the inner papilla, with continuous lamellae (compare with Plate 3, figure 4 and Plate 4, figure 6) and the lower part of the figure is the central area of the inner papilla, with a cavity (compare with Plate 3, figure 2 and Plate 4, figure 8). One can see, from the top to the bottom of the figure: a. an inner portion of the outer exospore; b. a less electron-dense inner exospore that is splitting into lamellae of the inner papilla; c. a basal lamina; d. more electron-dense elements that form a mesh on the inner surface of the basal lamina; and e. a megaspore lumen. Scale bar 1 μm .

(Plate 2, figure 2). A FIB-SEM study of palynological objects yields very good images, which are fully suitable for our purposes, but FIB-SEM is not as easily accessible a tool as conventional SEM, and the preparation of the material and handling of the microscope seem quite complicated.

The comparison between the two variants of embedding we tried shows that semithin sections of megaspores of the same taxon made with PVP-sucrose and epoxy resulted in comparable images when observed under SEM (Plate 4, figures 3, 5, 7 and Plate 4, figures 2, 4, 6, 8 respectively). We are satisfied with the information we can get via observation under SEM sections, prepared using both PVP-sucrose and epoxy media. However, we do not recommend PVP-sucrose medium because of the unstable properties of blocks and constant and

unpredictable difficulties during cutting. In addition, variations of orientation of PVP-sucrose blocks are limited: the drop hardens into a half-finished pyramid, which can be cut only transversely. It is unsuitable for flat fossil megaspores, which usually tend to stay in the block more or less horizontally. On the other hand, if we use epoxy medium, one and the same specimen can be studied in semithin (with SEM) and ultrathin (with TEM) sections, which can be important when only a few specimens are available for study, and also in the case of dispersed palynological objects of an unclear affinity. We think that this is the most suitable way to work: to embed the object and cut it by turns in semithin and ultrathin sections, which are then observed under SEM and TEM, respectively. Depending on the purposes of a study and a particular object, the thickness of semithin sections can

be changed (and, if so, the duration of treatment in Maxwell's solution also will be changed).

It should be stressed that it is not a sole study of semithin sections under SEM that we believe to be the most suitable for adequate reconstruction of the inner structure of relatively large palynological objects, but only such a study in combination with a study of ultrathin sections in TEM. SEM 3-D images of semithin sections help to understand 2-D TEM images of ultrathin sections of the same object, and the final reconstruction becomes more profound. A true organisation can be directly observed for some structural elements, for which several variants of reconstruction were conceivable on the basis of TEM data (e.g. tabulate elements with appendages in megaspores of *Grandispora ciliata* and inner papillae in megaspores of *Otynisporites tuberculatus*). The most suitable objects for such a SEM/TEM combined study are relatively large palynological objects such as megaspores (Zavialova & Turnau 2012; present paper) or large prepollen (e.g. Millay et al. 1980; Drinnan & Crane 1994), particularly if their structural elements are not too densely packed. It also seems that the method will yield good results in studies of saccate pollen and cavate spores. The application of both TEM and SEM is necessary because of several limitations of SEM. The resolution of SEM is lower than that of TEM. SEM does not allow one to recognise differences in electron density, and, therefore, boundaries between sublayers are indiscernible (ectexine/endexine and outer/inner exospore, etc.). If structural elements are too densely packed, the ultrastructure will not be correctly visible.

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Author biographies

NATALIA ZAVIALOVA is the head of the Laboratory of Palaeobotany at the A.A. Borissiak Palaeontological Institute, Russian Academy of Science. Natalia graduated from the M.V. Lomonosov Moscow State University, where she defended her thesis *Morphology and ultrastructure of fossil pollen grains from the upper Permian deposits of Viatka River Upstream and the Lower Jurassic deposits of Western Siberia*. Her palynological interests include the morphology and ultrastructure of fossil exines from various intervals, such as Late Palaeozoic and the Mesozoic.

EUGENY KARASEV was appointed as a senior researcher in the Laboratory of Palaeobotany at the A.A. Borissiak Palaeontological Institute, Russian Academy of Science in 2005. Eugeny received his PhD for a thesis entitled *The change of plant assemblages in the transitional stratigraphic interval at the Permian-Triassic boundary of the Moscow syncline*. His research focuses on the macroflora, dispersed leaf cuticles and megaspores from the Late Permian and Early Triassic of the Northern Hemisphere as well as palaeoclimate and plant biodiversity changes associated with the Permian–Triassic boundary. He administers the web site of the laboratory (<http://www.paleobotany.ru/>).

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